Expression of tau protein in non-neuronal cells: microtubule binding and stabilization

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Summary

The microtubule-associated protein tau is a developmentally regulated family of neuronal phosphoproteins that promotes the assembly and stabilization of microtubules. The carboxy-terminal half of the protein contains three copies of an imperfectly repeated sequence; this region has been found to bind microtubules in vitro. In addition, a fourth copy of the repeat has been found in adult-specific forms of tau protein. To examine the structure and function of tau protein in vivo, we have transiently expressed fetal and adult forms of tau protein and tau protein fragments in tissue culture cells. Biochemical analysis reveals full-length products with heterogeneity in post-translational modification synthesized in the cells. Immunofluorescent staining of transfected cells shows that, under our conditions, sequences on both sides of the repeat region are required for in vivo microtubule co-localization. These additional regions may be required either for enhancing microtubule contacts or for proper protein folding in the cell. In our expression system, the bundling of cellular microtubules occurs only in transfections using four-repeat tau constructs; any four-repeat construct capable of binding is also able to induce bundling. Our data suggest that the presence of bundles is correlated with enhanced microtubule stability; factors that increase stability such as higher levels of tau protein expression or the presence of the fourth repeat, increase the fraction of transfected cells showing bundles. Finally, the presence of tau protein in the cell allows all interphase microtubules to become acetylated, a post-translational modification usually reserved for a subset of stable cellular microtubules.

Key words: transient transfections, microtubule bundling, microtubule stability, acetylated tubulin.

Introduction

Microtubules are ubiquitous structures in eukaryotic cells and perform a variety of cellular functions. The ability of microtubules to achieve such functional diversity has been thought to be partially provided through the assistance of various associated proteins. Tau protein is a microtubule-associated protein found primarily in neuronal tissue. It promotes microtubule assembly in vitro (reviewed by Olmsted, 1986) and stabilizes cellular microtubules when microinjected into cells (Drubin and Kirschner, 1986). Expression of tau protein has been correlated with the extension of neurites during the differentiation of pheochromocytoma cells (Drubin et al. 1985). Moreover, the presence of tau antisense in primary neuronal cell culture appears to block the development and maintenance of axon-like processes, suggesting a role for tau protein in the establishment of neuronal cell polarity (Caceres and Kosik, 1990; Caceres et al., 1991). In situ, tau protein is associated with axons (Binder et al., 1985; Kowall and Kosik, 1987; Brion et al., 1988; Trojanowski et al., 1989).

The earliest purification of tau protein revealed a family of related phosphoproteins (Cleveland et al., 1977). It is now clear that some of this heterogeneity is generated by alternative splicing (Himmler, 1989). In human, cDNA clones for six tau isoforms and their developmental expression have been described (Goedert et al., 1988; Goedert et al., 1989a,b). Human adult-specific forms are distinguished by the presence of one or more of exons 2, 3 or 10 (Goedert et al., 1989a,b; exon number as assigned by Himmler (1989) for bovine gene). The most striking feature of the primary structure of tau protein as predicted from cDNA clones is the presence of three evenly spaced, imperfectly repeated copies of an 18 amino acid sequence in the carboxy-terminal half of the protein. One or more copies of the repeat are able to bind to microtubules in vitro (Butner and Kirschner, 1991; Himmler et al., 1989; Lee et al., 1989). Not surprisingly, increasing the number of copies of the repeat increased the efficiency of microtubule binding (Butner and Kirschner, 1991; Lee et al., 1989). Synthetic one-repeat peptides also promote microtubule assembly; however, the required stoichiometry indicates that the peptides are much less
potent than intact protein (Ennulat et al., 1989; Joly et al., 1989). The high molecular weight microtubule-associated protein MAP2 shares about 50% amino acid homology with tau protein at the carboxy-terminal end 220 residues and also contains three repeats (Lewis et al., 1988). As expected, a fragment of MAP2 from this area coassembles with microtubules in vitro (Lewis et al., 1988).

The adult-specific exon 10 encodes a 31 amino acid sequence that contains a fourth copy of the microtubule binding repeat unit. The inclusion of this fourth repeat increases the activity of tau protein in in vitro microtubule assembly assays (Goedert and Jakes, 1990). The expression of a four-repeat tau protein in vivo results in the bundling of cellular microtubules (Kanai et al., 1989).

In this study, we examined the in vivo structure and function of fetal and adult tau protein. Transiently expressed tau protein and tau protein fragments were characterized using Western blot and immunoprecipitation protocols. The ability of the expressed protein to associate with cellular microtubules and to induce microtubule bundling were determined by immunofluorescence. Our results indicate that, under our conditions, sequences adequate for in vitro binding are not adequate for in vivo co-localization to microtubules. Furthermore, the presence of adult-specific tau protein can induce microtubule bundling; microtubule stability is also increased. Bundling and stability may be related, since no evidence for cross-linking of microtubules by tau protein was found. Lastly, the presence of tau protein correlated with the acetylation of all cellular microtubules, indicating enhanced stability for all microtubules.

**Materials and methods**

**Construction of expression plasmids**

The eukaryotic expression vector pECE (Ellis et al., 1986) was modified in the following manner to introduce a unique NcoI cloning site into its multiple cloning site. First, the sole NcoI site in pECE was removed by NcoI cleavage followed by fill-in and re-circularization. The plasmid was then cut with *NcoI* site in the polylinker site downstream from the SV40 early promoter, filled in, then ligated to an *NcoI* linker in order to introduce an *NcoI* site at this location. The sequence of the new multiple cloning site in this vector, named pECE*N*, was checked by DNA sequencing.

Most three-repeat human tau inserts were prepared by polymerase chain reaction (PCR) using p19tau plasmid DNA (Lee et al., 1989) as template. PCR primers contained restriction sites that enabled insertion into pECE*N*. pEn was prepared by cutting and recircularizing pEn123c. In some plasmids, translation was terminated by a stop codon in the primer; in other plasmids, translation was terminated by the stop codons in the vector. Table 1 lists all plasmids used in this study; tau sequences expressed and new sequences contributed by primers, linkers or vectors are shown. The longest sequence contributed in this manner was 11 residues and its presence did not correlate with any functional property of the expressed proteins.

pEn123c was constructed from pEn123c by replacing the tau cDNA carboxy-terminal 500 bp with the analogous 593 bp fragment from an adult human tau four-repeat cDNA clone (Mori et al., 1989) kindly provided by Dr. H. Mori (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). The resultant cDNA is a full-length four-repeat tau cDNA identical to a clone previously identified by Goedert et al. (1989a). pE1234c(487) and pE1234(487) were constructed by primers containing *NcoI* linker; in other plasmids, translation was terminated by the stop codons by PCR using pl9tau plasmid DNA as template. pEn123(4c) was prepared by cutting and recircularizing pEn123c. The remaining human four-repeat tau inserts were prepared by PCR from pEn123c. Polymerase chain reaction primers used to prepare inserts for insertion into pECE*N* were identical to those used for the three-repeat human tau inserts and, therefore, the starts and stops of expressed sequences are identical.

Because most constructs were made using polymerase chain reaction technology, we acknowledge the small probability of a base change occurring during synthesis. However, most of the DNA fragments prepared were fairly small and the biochemical characterization of expressed protein (see below) would detect the inadvertant production of a termination codon. Also, all of the constructs overlapped, so that if a single amino acid replacement were to alter dramatically the properties of the expressed protein, an inconsistency would be noted.

**Transfections**

CHO or 3T3 (NIH) cells were grown in α-MEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and seeded onto 12 mm coverslips in 24-well plates at a density of approximately 0.2 × 10⁴ /well. CHO cells were transfected by calcium phosphate precipitation using standard

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**Table 1. Eukaryotic expression plasmids**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>NH₂-terminal sequences from primers, linkers or vector</th>
<th>Tau sequence (residue no.)</th>
<th>COOH-terminal sequences from primers, linkers or vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEn123c/pEn1234c</td>
<td>–</td>
<td>1-328/283</td>
<td>–</td>
</tr>
<tr>
<td>pEn</td>
<td>–</td>
<td>1-163</td>
<td>–</td>
</tr>
<tr>
<td>pEn1</td>
<td>–</td>
<td>1-224</td>
<td>–</td>
</tr>
<tr>
<td>pEn123(921)/pEn1234(921)</td>
<td>–</td>
<td>1-307/338</td>
<td>–</td>
</tr>
<tr>
<td>pEn123(843)/pEn1234(843)</td>
<td>–</td>
<td>1-281/312</td>
<td>–</td>
</tr>
<tr>
<td>pEn123(4c)</td>
<td>–</td>
<td>1-285</td>
<td>–</td>
</tr>
<tr>
<td>pE123(517)/pE1234(517)</td>
<td>MG</td>
<td>173-307/338</td>
<td>TVPRGIRALDK*</td>
</tr>
<tr>
<td>pE123(487)/pE1234(487)</td>
<td>MAG</td>
<td>164-307/338</td>
<td>TVPRGIRALDK*</td>
</tr>
<tr>
<td>pE123c(487)/pE1234c(487)</td>
<td>MAG</td>
<td>164-352/383</td>
<td>TVPRGIRALDK*</td>
</tr>
<tr>
<td>pE123c(517)/pE1234c(517)</td>
<td>MG</td>
<td>173-352/383</td>
<td>TVPRGIRALDK*</td>
</tr>
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</table>

*Indicates STOP codon.
techniques (Ausubel et al., 1989). 3T3 cells were transfected by calcium phosphate precipitation as modified by Chen and Okayama (1988). A 2 μg sample of plasmid DNA was added per well. Nocodazole treatment of the transfected cells was performed by adding nocodazole (Aldrich Chemical Co., Milwaukee, WI) to a final concentration of 3.3 μM, then incubating for the times indicated. Triton extractions were performed by first washing cells with extraction buffer minus Triton at 37°C, then incubating with extraction buffer (80 mM Pipes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 0.1% Triton-X100, 30% glycerol) at 37°C (Solomon et al., 1979). Extraction buffer was then removed and cells washed with extraction buffer minus Triton, then with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM PO₄, pH 7.4) before fixation. The time of extraction had been determined by inspecting the cells after extraction using anti-tubulin staining and by immunoblotting Triton-soluble and -insoluble fractions with anti-tubulin. Over extraction (1 min for 3T3 or 2 min for CHO cells) resulted in a large increase in Triton-soluble tubulin as detected by immunoblot and ruptured cytoskeletal network as detected by immunofluorescence. Therefore, extraction times of 0.5 min and 1 min were chosen for 3T3 and CHO, respectively. Cells were judged to be fully extracted, since non-cytoskeletal tau fragments were no longer detectable by immunofluorescence.

Immunofluorescence
Cells were fixed with 0.3% glutaraldehyde in 80 mM Pipes, pH 6.8, 1 mM MgCl₂, 5 mM EGTA, 0.5% NP40 for 10 minutes at room temperature (Drubin and Kirschner, 1986). After washing with PBS, cells were incubated with 10 mg/ml NaBH₄ for 7 minutes, washed with PBS, then incubated with 0.1 M glycine for 20 minutes. After washing with PBS, cells were incubated with methanol for 10 minutes. The initial step of this protocol combines permeabilization and fixation, because performing permeabilization first results in the extraction of tau protein from the detergent-insoluble cytoskeleton. Since full-length tau protein remains bound to microtubules under this fixation protocol, we chose to compare all constructs using these identical conditions. Microtubules were visualized by staining with the monoclonal antibody 6-11B-1 against acetylated tubulin kindly provided by Dr. G. Piperno (ICN Biochemicals, Inc., Irvine, CA) at 1:500 for 15 minutes at room temperature. Coverslips were mounted in 1 mg/ml p-phenylenediamine, 90% glycerol (Johnson and Araujo, 1981). Cells were photographed using ×63 Neofluar lens on a Zeiss Axioskop.

Bundling efficiency was determined by scoring 100-400 transfected cells for bundles. A cell was scored positive for bundling if one or more obviously thick, dense bands of microtubules were observed in the cell (see Fig. 5, below). Western blotting and immunoprecipitation
Cell lysates for Western blot analysis were prepared from transfected 3T3 cells by using 60 μl boiling of Laemmli SDS sample buffer (Laemmli, 1970) to scrape transfected cells from 24-well plates. Lysates were electrophoresed on 7.5% to 15% gradient gels, then transferred to Immobilon (Millipore Corp., Bedford, MA). The blot was probed with affinity-purified antibody to tau protein and 125I-labeled goat anti-rabbit secondary antibody (ICN Radiochemicals, Irvine, CA).

An estimation of the average amount of tau protein synthesized per transfected cell was made on the basis of the transfection efficiency and the amount of tau protein detected by Western blotting. The quantity of tau protein present in a harvested cell lysate (2×10⁶ cells) was calculated by excising the Immobilon band corresponding to the expressed protein, counting the 125I-labeled goat anti-rabbit secondary antibody bound, and converting the cts/min to protein, using Immobilon bands from adjacent lanes processed in parallel, which contained known amounts of fetal brain tau protein or Escherichia coli tau protein. The average amount synthesized per transfected cell equaled quantity in lysate divided by number of transfected cells (2×10⁶ × transfection efficiency; transfection efficiency was determined by a parallel transfection processed for immunofluorescence). Because the tau polyclonal antibody used to probe the blot was derived against brain tau protein, the reactivity of the antibody to the standard brain tau protein was greater than the reactivity to E. coli-synthesized tau protein, which lacks phosphorylated epitopes (e.g. 1 ng E. coli protein corresponded to 420 cts/min while 1 ng brain protein corresponded to 1516 cts/min). Since it is not known whether the reactivity of 3T3 expressed tau protein more closely resembles that of brain protein or E. coli protein, the amount of protein expressed is given as a range, using E. coli standard for the higher estimate, brain protein for the lower estimate. While in theory, a monoclonal antibody would have reacted to tau from all sources equally, in practice this did not work, since the monoclonal we tried was unable to yield a signal above background in detecting tau in transfected cell lysates. A comparison of the reactivity of the two antibodies to various quantities of E. coli tau protein showed the signal from the tau monoclonal (SE2) to be 5- to 10-fold less than that from the affinity-purified tau polyclonal antibody, respectively.

For two-dimensional gel analysis, transfections were performed in 6-well plates using 5 μg DNA per well. Cells were harvested by scraping with 80 μl boiling lysis buffer (0.5% SDS, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl). Samples were electrophoresed on NEPHGE gels (O'Farrell et al., 1977) using pH 3-10 Ampholines. The second dimension was 10% SDS/polyacrylamide gel, which was blotted and probed as described above. E. coli lysate containing full-length tau protein was prepared from E. coli BL21(DE3)LysS cells transformed with pET-3d expression plasmid (Studier et al., 1990) harboring full-length human three-repeat tau cDNA. Cell lysates for immunoprecipitation were prepared from 35S-methionine-labeled transfected 3T3 cells by using 80 μl lysis buffer supplemented with 2 mM EDTA, 2 mM PMSF (phenylmethylsulfoxyl fluoride) to scrape transfected cells from 6-well plates. (Each well had received approximately 175 μl of 35S-methionine for 4 hours prior to harvesting. Harvesting was performed 48 hours after DNA addition.) Lysates were boiled and then immunoprecipitated as described by Drubin et al. (1988) with the following modifications: lystate was preclared twice with Pansorbin and once with Protein A/Seaphorose beads (Pharmacia, Piscataway, NJ), Protein A/Seaphorose beads were used for adsorption of
antibody-antigen complexes, and beads were washed 5 times in NET (0.5% NP40, 50 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA) buffer prior to elution. Complexes were electrophoresed on 15% to 25% or 10% to 20% gradient gels. Gels were fluorographed prior to autoradiography.

Results
Full-length tau protein and tau protein fragments expressed in vivo
To study the in vivo structure and function of human tau protein, a panel of eukaryotic expression plasmids containing tau sequences was derived from both three-repeat and four-repeat tau cDNA clones (Fig. 1A,B). The four-repeat tau cDNA used was identical to the three-repeat clone except for the inclusion of tau gene exon 10, which encodes a fourth copy of the repeat. Most of the deletion constructs were made in the same manner for both the three-repeat and four-repeat constructs so that the starting and ending sequences of the expressed proteins were identical (Table 1). These plasmids were used to express transiently tau protein in 3T3 cells, which normally do not express tau protein.

To verify the presence of tau proteins in 3T3 cells, the transiently transfected cells were analyzed by Western blotting. Fig. 2 shows that detectable levels of full-length products are obtained from pEn123c, pEn123(921), pEn123(843), pEn, pEn123c, pEn1234c(921) and pEn1234c(843) (refer to Fig. 1 for tau sequences expressed). The estimated molecular masses were consistently higher than those calculated from primary structure data; this discrepancy may be due in part to phosphorylation of the protein (Lindwall and Cole, 1984) and has been noted for tau protein purified from brain as well. While multiple bands were seen in several lanes, it is unlikely that this reflected degradation, since there were no bands in common among the constructs, all of which overlapped in sequence.

To confirm the lack of degradation for the smaller constructs from the carboxy-terminal end, it was necessary to label the cells with 35S-methionine and immunoprecipitate tau protein as Western blotting was not sufficiently sensitive to visualize these proteins. Fig. 3 shows the labeled products immunoprecipitated from transiently transfected cells. Plasmids pE123c(487), pE123c(517), pE123(921), pE123(843), pE1234c(487) and pE1234c(517) exhibited protein patterns that suggest that intact protein is synthesized. The molecular masses calculated from size standards on the gel were much higher than those calculated from primary sequence data. For example, the two proteins in pE123c(487) (Fig. 3A, lane 2) average 32.5 kDa while the primary sequence calculation yields 20 kDa for residues 164 to 352. Therefore, besides indicating that intact protein is

![Fig. 1] (A) Schematic diagram of expression plasmid inserts from three-repeat tau cDNA. Each plasmid is constructed in expression vector pECEN+ as described in Materials and methods, and is predicted to express the indicated amino acids from tau protein. The human full-length three-repeat clone used was first reported by Goedert et al. (1988). The three 18 amino acid repeats identified in mouse tau protein (Lee et al., 1988) are located at residues 198-215, 229-246 and 261-278 in human; each repeat is marked with a short line. Microtubule binding capability, detected by immunofluorescence, is shown at the right. pE123(921), which encodes residues 164-307, was not included because no transfected cells were detected. (B) Expression plasmid inserts from four-repeat tau cDNA. Plasmids were constructed as described above. The human full-length four-repeat tau cDNA used is identical to the three-repeat cDNA above, except that it includes exon 10 (Himmler, 1989), which results in the insertion of 31 residues at residue 217 of the three-repeat sequence. The inserted sequence is shown by the heavy bar and contains the fourth repeat. Therefore, amino acids 248-383 of the four-repeat protein correspond to the three-repeat protein residues 217-352. The repeats are located at residues 198-215, 229-246, 260-277 and 292-309 and are marked with short lines. Microtubule binding and bundling capability for each construct are shown at the right. Relative bundling efficiencies are discussed in Results. (C) Diagram showing the role of various tau protein regions towards in vivo colocalization to microtubules. Numbers denote amino acid residues of three-repeat tau protein; numbers in parenthesis denote analogous residue numbers in four-repeat tau protein located downstream from exon 10, which inserts at residue 217. R indicates repeat region with the first repeat starting at residue 198 and the last repeat ending at 281(312).
Expression of tau protein in 3T3 cells

Fig. 2. Immunoblot of transiently transfected 3T3 cells. Cells were transfected and harvested as described in Materials and methods. Lysates were electrophoresed on a 7.5% to 15% gradient gel, blotted and probed with affinity-purified anti-tau followed by 125I-labeled goat anti-rabbit secondary. (A) Duplicate transfections using pEn123c (1-352), pEn123(921) (1-307), pEn123(843) (1-281) and pEn (1-163). Numbers at top indicate three-repeat tau residues expressed. (B) Transfections using pEn1234c (1-383), pEn1234(921) (1-338) and pEn1234(843) (1-312). Numbers at top indicate four-repeat tau residues expressed. Molecular mass markers (in kDa) are as indicated.

present, these data also suggest that post-translational modification in the 3T3 cells causes dramatic shifts in the gel mobility of these carboxy-terminal fragments. It is likely that these shifts are caused by phosphorylation; it has been suggested that phosphorylation may change the conformation of the molecule such that SDS binding is increased (Lindwall and Cole, 1984). Two-dimensional gel analysis shows in vivo expressed tau protein to be much more acidic than the identical protein synthesized in E. coli (data not shown).

In Fig. 3A, cells transfected with pE123c(517) (lane 3) express two forms that appear to differ in molecular mass by approximately 2.5 kDa. When the carboxy-terminal 45 amino acids was truncated (pE123(517), lane 5), heterogeneity was lost, since only one form was detected. This suggests that residues 308-352 contain a site that creates heterogeneity in the gel mobility of the protein. Indeed, Steiner et al. (1990) have reported that phosphorylation of serine residue 327 of tau protein results in a shift in the electrophoretic mobility of tau protein. However, other sites with this property exist, since doublet bands are also exhibited by constructs lacking this same region (e.g. pEn123(921), pEn1234(921)).

The difference in gel mobility between three-repeat fragments 164-352 and 173-352 (Fig. 3A, lanes 2, 3) is larger than one would expect from a nine amino acid difference. Since the 164-352 products (lane 2) are estimated to be 34 kDa and 31 kDa while the 173-352 products (lane 3) are estimated to be 26.3 kDa and 23.8 kDa, the inclusion of residues 164-172 in these three-repeat fragments results in a shift in the electrophoretic mobility of tau protein. However, other sites with this property exist, since doublet bands are also exhibited by constructs lacking this same region (e.g. pEn123(921), pEn1234(921)).

Fig. 3. Immunoprecipitation of 35S-labeled tau protein fragments from transiently transfected 3T3 cells. Immune complexes from 3T3 transfected cell lysates were prepared as described in Materials and methods. (A) A 15% to 25% gradient gel displaying products from transfections with three-repeat constructs. Lane 1 shows control immunoprecipitation from non-transfected cells. Lane 2 contains immunoprecipitated products from pE123c(487) transfection expressing residues 164-352, lane 3 pE123c(517) expressing residues 173-352, lane 4 pE123(487), and lane 5 pE123(517) expressing residues 173-307. Positions of bands marked with arrows (lane 2) correspond to molecular masses of 34 kDa and 31 kDa; bands marked with asterisks (lane 3) 26.3 kDa and 23.8 kDa; dark band in lane 5, 16 kDa. Molecular mass calculated from primary structure for 164-352 is 20 kDa, 173-352 19 kDa, and 173-307 14.4 kDa. (B) A 10% to 20% gradient gel displaying products from transfections with four-repeat constructs. Lane 1 shows immunoprecipitated products from pE1234c(487) transfection expressing residues 164-383, lane 2 pE1234c(517) expressing residues 173-383, and lane 3 non-transfected control. Positions of bands marked with arrows correspond to molecular masses of 35 kDa and 33 kDa. Molecular masses calculated from primary sequence data for residues 164-383 is 23.3 kDa and for 173-383 is 22.3 kDa. Molecular mass standards are as shown.
164-172 could affect the recognition of residue 173 by kinases). The contribution of residues 164-172 towards causing gel mobility shifts is diminished, however, when four repeats are present. Fig. 3B shows that four-repeat fragments 164-383 (lane 1) and 173-383 (lane 2) look identical.

We were unable to immunoprecipitate products for pE123(487) (Fig. 3A, lane 4), pE1234(487) and pE1234(517) (data not shown). Since these plasmids had transfection efficiencies of 0-1% as assessed by immunofluorescence (see below), we judged that very little protein was present. Plasmids whose products were detectable by Western blotting had transfection efficiencies of 20-40% while those detectable only by immunoprecipitation had efficiencies of 5-10%. Since the necessity to immunoprecipitate correlated with plasmids encoding carboxyl-terminal fragments, we surmised that these protein fragments or their mRNAs were susceptible to cellular degradation.

An estimate of the amount of full-length tau protein synthesized in transfected 3T3 cells was made by comparing the Western blot signal to the signal given by known quantities of tau protein (see Materials and methods). By dividing this number by the number of transfected cells in the culture, as determined in a parallel transfection, we estimate that an average of 0.5-2 picograms of full-length tau protein is synthesized in a transiently transfected cell.

Requirements for co-localization of tau protein and microtubules in vivo

The in vivo function of tau protein and tau protein fragments was assessed by examining transiently transfected CHO or 3T3 cells by immunofluorescence. The cells were fixed 24-48 hours after DNA addition and stained with antibodies to tau protein and tubulin, using double immunofluorescence to show the location of tau protein relative to the microtubules. Because the cells transiently express tau protein, the level of expression varies from cell to cell, depending on the number of gene copies present. In looking at many cells, we found that only the intensity of the pattern of staining varied with the level of expression; the pattern did not vary. A fraction of the transfected cells had no discernible staining pattern, perhaps due to the cell-cycle stage, and were not included in the analysis.

Fig. 4 shows tau protein and tubulin staining of CHO cells transfected with plasmids pEn123c, pEn and pE123. As expected, full-length tau (pEn123c) co-localizes with microtubules (Fig. 4a) while the amino-terminal fragment having no repeats (pEn) shows cytoplasmic rather than cytoskeletal staining (Fig. 4b). Surprisingly, cells transfected with pE123 did not show tau protein co-localizing to microtubules (Fig. 4c); this fragment contains the entire repeat region and was shown to bind to taxol-stabilized microtubules in vitro (Lee et al., 1989). Interaction with the cytoskeleton was confirmed by extraction with 0.1% Triton prior to fixation and immunofluorescence (conditions for extraction were derived as described in Materials and methods). Triton extraction removes soluble components from the cytoplasm, leaving intact microtubules unobscured by cytoplasmic staining (Solomon et al., 1979). This procedure did not reveal any fraction of cytoplasmically localized tau fragments binding to microtubules. Also, none of the constructs exhibiting microtubule localization showed significant loss of binding after Triton extraction.

Fig. 1 shows the microtubule localization results for our panel of constructs. From our data, we conclude that under our conditions of fixation, residues 164 to 307 of the three-repeat tau protein are required for microtubule association in the cell. For four-repeat tau constructs, deleting residues upstream from residue 338, which corresponds to three-repeat residue 307, also resulted in loss of binding. However, the required sequence at the amino-terminal side of the four-repeat
Microtubule bundling

Cells with full-length four-repeat tau protein (pE1234c) exhibited bundled microtubules similar to those reported in mouse L-cells by Kanai et al. (1989), where a rat full-length four-repeat tau cDNA was used. Bundles were most dramatically seen as sharp spikes emanating from the cell or as thick dense bands running along the edge of cells (Fig. 5). Kanai et al. (1989) have defined bundles as microtubule structures with 10-100 times the thickness of normal microtubules. In non-transfected 3T3 cells, microtubules did not coalesce into such structures. In contrast to the cytoplasmic versus cytoskeletal localizations described above, the occurrence of bundles appeared to be correlated with the level of expression of the four-repeat protein; bundles were never found in cells with faint tau staining. Transfections with four-repeat protein showed an average of 20% transfected cells containing bundled microtubules. In contrast, while the three-repeat construct consistently had a higher transfection efficiency than the four-repeat construct, only 1% of cells transfected with the three-repeat protein showed bundling. When non-transfected cells were scored for bundled microtubules, less than 0.25% were positive.

Using the collection of human tau constructs bearing four repeats, we found that every tau protein fragment capable of binding to microtubules also exhibited bundled microtubules (Fig. 1B). Therefore, the carboxy-terminal 45 residues and the amino-terminal 172 residues of tau protein are not required for this result.

Tau protein stabilized microtubules in cells

Drubin and Kirschner (1986) have shown that tau protein, when microinjected into cells, stabilizes microtubules against drug-induced depolymerization. We found that cells transfected with four-repeat tau cDNA were able to withstand more prolonged drug treatment than those transfected with three-repeat cDNA. Microtubule fragments were still visible in cells expressing four-repeat protein after 1 hour of incubation in 3.3 μM nocodazole (Fig. 6a) while none were present in the three-repeat protein transfectants (Fig. 6b) and non-transfected cells (Fig. 6c). After 3 hours of drug treatment, microtubules were absent in all cells.

The enhanced stability of tau-associated microtubules was further demonstrated by staining transfected cells with an antibody to acetylated tubulin (Piperno and Fuller, 1985). Microtubules containing acetylated-tubulin subunits have been identified as a stable subset of microtubules that persist after low-level nocodazole treatment (Piperno et al., 1987) and turn over less rapidly in the cell relative to dynamic microtubules (Schulze et al., 1987; Webster and Borisy, 1989). The top cell in Fig. 7b shows a typical acetylated tubulin pattern in a non-transfected cell. Looking at transfected cells that exhibited an interphase micro-
Regions flanking the repeat region contribute to microtubule co-localization in vivo

The structure and function of tau protein and MAP2 protein have been studied in vitro using protein fragments synthesized in E. coli or in rabbit reticulocyte lysate systems (Butner and Kirschner, 1991; Himmler et al., 1989; Lewis et al., 1988; Lee et al. 1989). These data suggest that sequences up to residue 190 on the amino-terminal side and sequences downstream from residue 281 on the carboxy-terminal side could be deleted without abolishing in vitro microtubule binding activity. However, our in vivo experiments indicate that additional sequences on each side of the repeat region are necessary for co-localization of tau protein to cellular microtubules. Butner and Kirschner (1991) also found in their in vitro binding study that sequences outside of the repeats (residues 92-190 or 289-352, numbered as in three-repeat tau) can increase the affinity of a tau fragment for microtubules 10- to 20-fold. With MAP2, Lewis et al. (1989) also found that including residues 145-193 or 292-352 qualitatively enhanced in vivo microtubule binding. Therefore, while the presence of three- to four-repeat motifs can result in 80-100% of the input tau fragment binding to microtubules 10- to 20-fold. With MAP2, Lewis et al. (1989) also found that including residues 145-193 or 292-352 qualitatively enhanced in vivo microtubule binding. Therefore, while the presence of three- to four-repeat motifs can result in 80-100% of the input tau fragment binding to microtubules 10- to 20-fold. With MAP2, Lewis et al. (1989) also found that including residues 145-193 or 292-352 qualitatively enhanced in vivo microtubule binding. Therefore, while the presence of three- to four-repeat motifs can result in 80-100% of the input tau fragment binding to microtubules 10- to 20-fold. With MAP2, Lewis et al. (1989) also found that including residues 145-193 or 292-352 qualitatively enhanced in vivo microtubule binding. 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As summarized in Fig. 1C, we find that including up to residue 164 on the amino side or down to residue 307 on the carboxy side of the repeats is sufficient to differentiate between in vivo binding and not binding in our system.

The role of the contributions made by the flanking
regions may be to enhance microtubule interaction by providing additional contacts that by themselves are insufficient to bind microtubules. These contacts may not be needed for in vitro binding because in vitro assays contain a vast excess of stabilized microtubules, which may drive the binding reaction. Our in vivo data reflect the cellular conditions normally encountered by tau protein. Besides smaller amounts of microtubules, there may also be the presence of competing endogenous microtubule binding proteins. Alternatively, the flanking sequences may be necessary for efficient folding of tau fragments. While this may not have been critical for an in vitro assay with purified components, in vivo, an improperly folded protein may bind elsewhere or lack the higher binding affinity required to yield detectable localization to microtubules. While our fixation conditions may reflect a 'stringent' binding requirement, the existence of these non-repeat regions that enhance binding may suggest the existence of specific functions of tau that require a higher binding affinity than that solely necessary to bind to microtubules in vitro.

Microtubule bundling in the presence of tau and MAP2

Our results show that the expression of the fourth-repeat leads to a discernible functional difference in the microtubule organization and stability in cells. In our expression system, every four-repeat microtubule-binding tau construct was more effective in inducing microtubule bundling than the analogous three-repeat construct. The immunofluorescent staining of cells transiently transfected with a fetal mouse tau expression plasmid, as reported by Lewis et al. (1989), is consistent with our results in that very little bundling is exhibited by three-repeat forms. However, they found that MAP2, which also contains three repeats, induces bundling. Since MAP2 and tau protein share only 50% homology in the carboxy-terminal 220 residues, it is likely that the sequence differences account for the difference in bundling capability. Moreover, MAP2 is more basic than tau protein in this region and, since the interaction of these proteins with microtubules has been postulated to be one of an ionic nature (Vallee, 1982), MAP2 would be more effective than tau protein. It has been reported that MAP2 promotes assembly, nucleates microtubules and stabilizes microtubules more efficiently than tau protein (Sandoval and Vandekerckhove, 1981).

Recently, Lewis and Cowan (1990) have reported that the area responsible for bundling microtubules in MAP2 is the area homologous to amino acids 313-335 of four-repeat tau protein (282-304 in three-repeat) and that MAP2 lacking this region is still able to bind microtubules. In tau protein, this area is required for both microtubule bundling and stabilization of microtubules. In our study, the constructs with the highest bundling efficiencies were pEnl234c and pEnl234(921). Among the constructs capable of inducing bundles, these two also exhibited the highest transfection efficiency and expressed picogram levels of protein in cells, allowing for detection by Western blotting. Lastly, the propensity for microtubule bundles to form in the cell may also depend on the cell type employed, since bundling may require re-modeling of existing cytoskeleton; the ease with which the existing microtubules are re-arranged may vary from cell to cell type. The expression of tau protein in baculovirus-infected Sf9 cells provides an example of an extremely high level of tau expression (greater than 10-fold of that of transfected cells) achieved in the presence of a pool of unpolymerized tubulin (Knops et al., 1991). In this system, microtubule bundles are formed in 100% of the infected cells using either three- or four-repeat tau-expressing virus. This result stresses the difficulties in comparing tau protein's bundling activity where different expression systems have been used and indicates that valid comparisons between different isoforms or different fragments must be made within a single expression system. Moreover, it also shows that a heightened level of protein expression can compensate for a lowered binding affinity towards inducing microtubule bundling.

Microtubule stabilization and bundling

The deletion analysis of the four-repeat tau protein indicates that sequences upstream from amino acid 173 and downstream from 338 are not required for microtubule bundling. We propose that bundling is very likely the result of enhanced stabilization of microtubules, on the basis of the following reasons: (1) the areas of tau protein required for bundling correlate with those involved in microtubule binding. (2) Bundled microtubules are seen mainly in cells with a high level of tau expression. (3) Tau expression. (3) The presence of the required fourth repeat increases the stability of the microtubules, as shown by nocodazole resistance. The number of cells showing bundled microtubules in a given transfection would be related to the association constant of the stabilizing protein and the level of expression. It is probable that, for the induction of bundles, a heightened level of expression may compensate for a lower association constant and vice versa. Kanai et al. (1989) have also suggested that bundling is increased in cells with a high level of tau protein expression, on the basis of a comparison of transient and stable expression data. In our study, the constructs with the highest bundling efficiencies were pEnl234c and pEnl234(921). Among the constructs capable of inducing bundles, these two also exhibited the highest transfection efficiency and expressed picogram levels of protein in cells, allowing for detection by Western blotting. Lastly, the propensity for microtubule bundles to form in the cell may also depend on the cell type employed, since bundling may require re-modeling of existing cytoskeleton; the ease with which the existing microtubules are re-arranged may vary from cell to cell type. The expression of tau protein in baculovirus-infected Sf9 cells provides an example of an extremely high level of tau expression (greater than 10-fold of that of transfected cells) achieved in the presence of a pool of unpolymerized tubulin (Knops et al., 1991). In this system, microtubule bundles are formed in 100% of the infected cells using either three- or four-repeat tau-expressing virus. This result stresses the difficulties in comparing tau protein's bundling activity where different expression systems have been used and indicates that valid comparisons between different isoforms or different fragments must be made within a single expression system. Moreover, it also shows that a heightened level of protein expression can compensate for a lowered binding affinity towards inducing microtubule bundling.

Our proposal that tau induces microtubule bundling by stabilizing microtubules is also supported by our finding that the minimal unique tau sequence required for binding and bundling is too small to span the 20-25 nm distance between bundled microtubules, especially if the entire repeat region is bound to a single microtubule. The possibility that a single repeat region binds to two microtubules would require that only a
single repeat be in contact with each microtubule; given the much reduced binding affinity of single repeat units (Butner and Kirschner, 1991), it seems unlikely that such a configuration would be physiologically relevant. The report that taxol produces similar microtubule bundles (Chapin et al., 1991) is consistent with our findings. With the increased stability of microtubules in taxol-treated or tau-transfected cells, other cellular factors may participate in the bundling process. Vallee (1990) has raised the possibility that cells transfected with MAP2 may over-express other MAPs that may be involved in bundling. Another possibility is that stabilized microtubules may have new physicochemical properties at their surfaces that make them self-adherent.

If microtubule bundling does not require other proteins, it should be reproducible in vitro. While we have been able to obtain microtubule bundles in vitro using E. coli-synthesized tau protein in a centrosome-mediated microtubule regrowth assay (Brandt and Lee, unpublished data), the relationship between these bundles and those seen in 3T3 cells remains to be shown.

The presence of tau protein is correlated with the acetylation of all cellular microtubules

The acetylation of microtubules has been postulated to be the consequence of microtubule stabilization (Piperno et al. 1987; Schulze et al., 1987; Webster and Borisy, 1989). Therefore, one might expect microtubules stabilized by tau protein to be acetylated. In our transfections, we found many transfected cells exhibiting an acetylated tubulin staining pattern coinciding with the general cytoplasmic interphase microtubule array as detected by tau staining. This supplies new evidence that tau protein stabilizes microtubules in vivo, leading to the acetylation of all microtubules in the cell; other lines of evidence have been based on the use of microtubule depolymerizing drugs (Drubin and Kirschner, 1986). However, transfected cells with acetylated tubulin staining patterns resembling those found in non-transfected cells were also present; in these cells, only a subset of microtubules were acetylated. This suggests that microtubule acetylation is regulated by other factors in addition to stability, as conferred by tau protein association. Webster and Borisy (1989) have found that acetylated microtubules are absent among prophase microtubules and that their reappearance lags behind that of the cytoplasmic microtubules after the completion of mitosis. This suggests that acetylation may be cell cycle regulated and that our transfected cells have a distribution of acetylated tubulin patterns due to asynchrony in the culture.

Conclusions

In summary, our results show that regions outside the repeat region of tau protein influence the ability of the protein to bind to microtubules in vivo. We define regions flanking the repeats that are required for in vivo binding under our conditions. The requirement for additional sequence on each side of the repeat region may be related to protein folding and/or to additional microtubule contact sites. The presence of tau protein in the cell is correlated with the acetylation of all cellular microtubules, possibly in a cell cycle-dependent manner. When tau protein with four-repeats was expressed, thick dense bands of microtubule bundles were observed in cells. Deletion analysis did not reveal any unique sequence associated with bundling; four-repeat constructs capable of binding also induced microtubule bundling. Also, microtubules associating with four-repeat tau in vivo were found to have greater stability, as assessed by nocodazole resistance, than those associating with three-repeat tau. Our data suggest that microtubule bundling is correlated with increased stability of microtubules. Since the fourth repeat is found only in adult isoforms of tau protein, our work provides evidence that the developmental regulation of tau protein heterogeneity bears functional implications for cellular microtubules.

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References


Expression of tau protein in 3T3 cells


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